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Anti-Feline Immunodeficiency Virus (FIV) Soluble Factor(s) Produced from Antigen-Stimulated Feline CD8$^+$ T Lymphocytes Suppresses FIV Replication

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Feline immunodeficiency virus (FIV) causes AIDS-like symptoms in infected cats. Concanavalin A (ConA)-stimulated peripheral blood mononuclear cells (PBMC) from chronically FIV strain PPR-infected cats readily expressed FIV. In contrast, when PBMC from these animals were stimulated with irradiated, autologous antigen-presenting cells (APC), at least a 10-fold drop in viral production was observed. In addition to FIV-specific cytotoxic T lymphocytes, anti-FIV activity was demonstrated in the cell-free supernatants of effector T lymphocytes stimulated with APC. The FIV-suppressive activity was induced from APC-stimulated PBMC of either FIV-infected or uninfected cats but not from ConA-stimulated PBMC. Suppression of FIV strain PPR replication was observed for both autologous and heterologous feline PBMC, was dose dependent, and demonstrated cross-reactivity and cell specificity. It was also demonstrated that the anti-FIV activity originated from CD8$^+$ T lymphocytes and was mediated by a noncytolytic mechanism.

Feline immunodeficiency virus (FIV) is a T-lymphotropic lentivirus in the family *Retroviridae* (35). Infection of cats with FIV causes transient fever, leukopenia, generalized lymphadenopathy, and finally an immunodeficiency-like syndrome, such as stomatitis, upper respiratory disease, neurological disease, or lymphoma (16, 35, 50). FIV infects both CD4$^+$ and CD8$^+$ T lymphocytes (9) and induces the characteristic inversion of CD4$^+$/CD8$^+$ T-cell ratios (1, 16, 31, 42). As with human immunodeficiency virus (HIV) infection in humans (7, 26, 46), following infection of cats with FIV, cytolytic and noncytolytic cellular immune mechanisms are suggested to participate in the control of virus replication in vivo (3, 10, 23, 40).

Major histocompatibility complex-restricted FIV- and HIV-specific cytotoxic T lymphocytes (CTL) are predominantly composed of CD8$^+$ T lymphocytes (7, 40). CTL-mediated cellular immunity has been suggested to control the initial viral infection prior to the production of virus-specific antibody (3, 26). It has also been suggested that CTL activity correlates with disease progression in HIV-infected patients (20, 37). HIV type 1-infected individuals exerting active CTL responses showed rapidly reduced plasma viremia and virus replication. In contrast, patients with low or undetectable CTL responses did not show control of virus replication and progressed to the AIDS state. FIV-infected cats or cats vaccinated with inactivated FIV have been shown to produce FIV-specific CTL responses (3, 18, 28, 41, 44). FIV-specific CTL also are suggested to participate in establishing protective immunity in cats (21, 45).

CD8$^+$ T lymphocytes from HIV-infected subjects have also been shown to inhibit viral replication without cytotoxicity in CD8$^+$ T-cell-depleted peripheral blood mononuclear cells (PBMC) (46). Furthermore, this noncytolytic mechanism could be reconstituted following the addition of CD8$^+$ T cells to infected CD4$^+$ T lymphocytes (48). The soluble anti-HIV factor present in cell-free supernatants of CD8$^+$ T cells was later designated CD8$^+$ T-cell antiviral factor (29, 47). The presence of similar soluble antiviral factors was demonstrated for CD8$^+$ T cells of simian immunodeficiency virus-infected African green monkeys and HIV type 2-infected baboons (5, 17). In addition to the uncharacterized T-cell antiviral factor, interleukin 16 (IL-16) and several chemokines have been identified as HIV-suppressive factors (2, 6, 13, 33). It appears that the antiviral activities derived from CD8$^+$ cells consist of multiple independent factors (38, 39). Like the cytolytic antiviral activity of CTL, the noncytolytic antiviral activities of CD8$^+$ T cells were associated with the clinical stages of HIV and FIV infections (4, 10). CD8$^+$ T lymphocytes from HIV-infected long-term survivors very efficiently suppressed HIV replication, but CD8$^+$ T cells from AIDS patients did not inhibit viral replication (4). Some infected cats, showing no evidence of seroconversion or FIV infection, had strong CD8$^+$ T-cell-mediated activity that readily inhibited FIV replication (10).

In the present study, we demonstrated that PBMC with consistent FIV-specific CTL activity produced an anti-FIV soluble factor(s) following stimulation with FIV-infected and irradiated antigen-presenting cells (APC). The anti-FIV factor(s) suppressed viral replication in PBMC by a noncytolytic mechanism.

MATERIALS AND METHODS

Experimental animals. Specific-pathogen-free cats purchased from Harlan Sprague-Dawley, Madison, Wis., or Liberty Laboratories, Liberty Corner, N.J., were serologically negative for feline leukemia virus. Cats were housed in an specific-pathogen-free environment at the Laboratory Animal Research and Resources Facility, Texas A&M University, College Station. Cats AUO2, AUO3, AWF1, AZV2, OLQ5, E238, E284, 306, and 308 were chronically infected with FIV strain PPR (FIV-PPR). Cats AUS3, E266, OAE5, OLM6, and OLQ4, sham inoculated with saline solution, were used as negative control cats.

Virus. FIV-PPR was propagated in feline PBMC, and FIV strain Pet (FIV-Pet) was propagated in Crandell feline kidney (CrFK) cells (36). After 7 to 10 days of infection, virus replication was evaluated with an FIV capsid antigen (p24) detection enzyme-linked immunosorbent assay (ELISA) kit (IDEXX, Portland, Maine). Supernatants with an optical density (OD) of more than 3.5 were collected, and these stocks were stored at −70°C.

Cell culture. Feline PBMC were isolated from EDTA (K_3)-treated whole blood by Histopaque-1077 (Sigma, St. Louis, Mo.) density gradient centrifugation. PBMC were cultured as described previously with RPMI 1640 (Gibco BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (FBS), 50 μg of gentamicin (Gibco BRL) per ml, 5×10^{-5} M 2-mercaptoethanol (Gibco BRL),

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2 mM L-glutamine (Gibco BRL), and 100 U of human recombinant IL-2 (hr IL-2) (Collaborative Biomedical Products, Bedford, Mass.) per ml (40). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every 3 to 4 days. FIV replication in the culture supernatants was determined by detection of FIV capsid antigen with the ELISA kit. Phenotypes of T cells were analyzed by flow cytometry as described previously (28). FIV-Pet-infected CrFK cells were cultured in complete Dulbecco modified Eagle medium supplemented with 10% FBS and 50 μg of gentamicin per ml.

Stimulation of effector cells. Feline PBMC were stimulated with 5 μg of concanavalin A (ConA) mitogen per ml for 3 days. The stimulated lymphoblastoid cells were infected with 0.5 ml of FIV-PPR stock for 30 min at 37°C and cultured in complete RPMI 1640. After 6 days of culturing, virus infection was verified with the capsid antigen ELISA kit. These FIV-infected cells were used as APC for the stimulation of effector cells and as target cells in CTL assays. FIV-infected cells were inactivated by irradiation (10,000 rads from a ^{60}Co source) and used as autologous APC by coculturing with effector cells (40). Effector cells (10^6 cells/ml) were stimulated every 3 to 4 days for the first 10 days in the presence of only APC (1×10^5 to 2×10^5 cells/ml), without ConA or hr IL-2. On day 10, viable effector cells were isolated by Histopaque-1077 gradient centrifugation, resuspended in complete RPMI 1640 supplemented with 100 U of hr IL-2 per ml, and cultured with APC for an additional 4 days. After 2 weeks of stimulation with APC, CTL responses of effector cells were determined with FIV-infected target cells.

Cytotoxicity assay. CTL assays were performed as previously described but with modifications (28, 40). Viable FIV-infected cells were isolated by Histopaque-1077 gradient centrifugation. Purified FIV-infected cells (10^6 for each group) were washed twice with complete RPMI 1640, and the supernatants were removed from the cell pellets. The cells were resuspended in 100 μl of complete RPMI 1640, and 5 μCi of indium-111 oxine (Medi-Physics Amersham, Inc., San Antonio, Tex.) was added to each cell group. The cells were incubated at 37°C for 30 min. ^{111}In-labeled cells were washed four times with complete RPMI 1640 and resuspended in 10 ml of complete RPMI 1640. CTL assays were done in triplicate at various effector/target (E/T) ratios. Target cells (5×10^3) in 100 μl of complete RPMI 1640 were placed in each well of V-bottom 96-well tissue culture plates (Costar, Cambridge, Mass.). Effector cells (100 μl) were added to each well containing target cells at the appropriate E/T ratio. Spontaneous release was measured for wells receiving 100 μl of complete RPMI 1640, and maximum release was measured for wells receiving 100 μl of 3% Triton X-100 instead of effector cells. The plates were centrifuged at $125 \times g$ for 4 min, incubated at 37°C for 4 h, and then centrifuged at $450 \times g$ for 5 min. A 100-μl portion of supernatant was taken from each well, and counts per minute were counted with a gamma radiation counter (Cobra Auto-Gamma Counter; Packard Instrument Company, Meriden, Conn.). The percent specific cytotoxicity for triplicate samples was calculated as follows: percent cytotoxicity = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100. The percentage of spontaneous release/maximum release was less than 20%.

Culture supernatants from effector cells. Cell-free supernatants were collected from APC-stimulated effector cells induced from PBMC of FIV-PPR-infected and uninfected cats. The supernatants were centrifuged at 25,000 rpm for 2 h (Beckman Instruments, Palo Alto, Calif.) to pellet the residual viral particles (11) before passage through 0.2-μm-pore-size filters. The amount of FIV capsid antigen (p24) remaining in the supernatants after ultracentrifugation was negligible (data not shown). The supernatants were stored at −70°C.

Phenotype enrichment. The panning method was used for enrichment of $CD8^+$ and $CD4^+$ T cells from freshly isolated PBMC or APC-stimulated effector cells (49). Briefly, 10^7 PBMC were incubated on ice for 30 min with 2 ml of phosphate-buffered saline (PBS) containing 10 μg of mouse anti-cat $CD4^+$ or anti-cat $CD8^+$ monoclonal antibody (Southern Biotechnology Associates, Inc., Birmingham, Ala.) per ml. The cells were washed twice, resuspended in 4 ml of PBS containing 1% FBS, and incubated on a petri dish coated with goat anti-mouse immunoglobulin G monoclonal antibody (Southern Biotechnology Associates) for 2 h at 4°C. The goat anti-mouse immunoglobulin G-coated petri dish was prepared by incubating 10 μg of monoclonal antibody per ml in PBS overnight at room temperature and washing with cold PBS. Plates were incubated with PBS containing 1% FBS prior to use to prevent nonspecific binding of PBMC. Unbound $CD8^+$ or $CD4^+$ cells were collected and cultured in complete RPMI 1640. The purity of depleted cells obtained by this method was greater than 95%, as determined by flow cytometry (data not shown).

Human chemokines. Human recombinant β-chemokines, MIP-1α, MIP-1β, and RANTES (PeproTech, Inc., Rocky Hill, N.J.), were diluted in complete RPMI 1640 at a concentration of 1,000 ng/ml. A human recombinant α-chemokine, SDF-1α (PeproTech), was diluted in complete RPMI 1640 at a concentration of 4,000 ng/ml. The diluted chemokines were added to FIV-PPR-infected feline PBMC at a 1:1 ratio of cell culture medium to chemokine solution, and their suppressive activities were compared with that of the supernatant of APC-stimulated feline effector cells.

Statistical analysis. The differences in FIV replication and cell viability between infected control cells and anti-FIV factor(s)-treated cells were analyzed with a two-tailed Student *t* test (32). Statistical significance was set at a *P* value of <0.05 or, in general, 65% or less virus expression than in untreated controls.

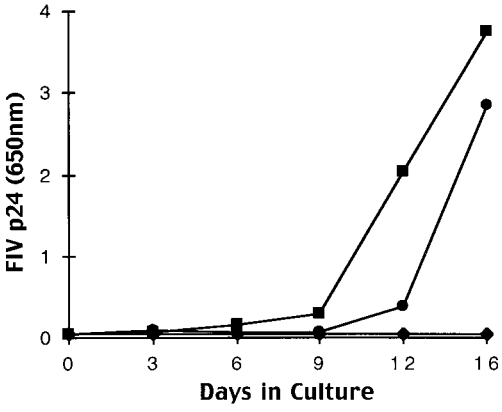

FIG. 1. Kinetics of FIV replication in PBMC of infected cats. PBMC of uninfected and FIV-infected cats were cultured at a concentration of 5×10^5/ml in RPMI-1640 supplemented with 100 U of hr IL-2 per ml after ConA stimulation for the first 3 days. The culture supernatants were harvested on the indicated days of culturing. FIV replication was measured by use of an FIV capsid antigen (p24) ELISA. Data are for uninfected cat AUS3 (diamonds) and FIV-infected cats 306 (circles) and 308 (squares).

RESULTS

Kinetics of FIV replication in PBMC. FIV infects both feline $CD4^+$ and feline $CD8^+$ T cells (9, 14, 15). In this report, the kinetics of FIV-PPR isolation were determined with ConA-stimulated, unfractionated PBMC and $CD4^+$ and $CD8^+$ T cells from experimentally FIV-PPR-infected cats. FIV-PPR could be isolated from PBMC of FIV-PPR-infected cats 306 and 308 (Fig. 1). No viral antigen was detected in cultured PBMC of uninfected cat AUS3 (Fig. 1). However, in cultured PBMC of both FIV-PPR-infected cats, FIV replication could be detected within 9 to 12 days of culturing. The production of FIV antigen rapidly increased until day 16 of culturing, the last day examined. This study demonstrated that FIV-PPR could be isolated from ConA-stimulated, unfractionated PBMC of infected cats even without the removal of $CD8^+$ T lymphocytes. FIV-PPR replication in cultured $CD4^+$ and $CD8^+$ T cells was examined to confirm the cell tropism of FIV. The $CD4^+$ T lymphocytes of cat 306 produced 5.8-fold more FIV than the $CD8^+$ T lymphocytes of the same cat after 15 days of culturing (Fig. 2). In contrast, the $CD8^+$ T lymphocytes of cat 308 produced more FIV than the $CD4^+$ T lymphocytes of the same cat (Fig. 2). In addition, both $CD4^+$ and $CD8^+$ T cells of cat 308 reproducibly expressed more viral antigen than those of cat 306 under identical culture conditions.

Development of FIV-specific CTL. The presence of FIV-specific CTL was verified by use of PBMC from FIV-PPR-infected cats. PBMC of infected cats 306 and 308 and uninfected cats E266 and AUS3 were stimulated for 2 weeks with autologous, irradiated FIV-PPR-infected cells. FIV-infected autologous PBMC were used as target cells. Antigen-stimulated effector cells of FIV-infected cats 306 and 308 demonstrated 30% lysis of target cells at an E/T ratio of 100:1 (Fig. 3A) and 15% lysis of target cells at an E/T ratio of 80:1 (Fig. 3B), respectively. In contrast, there was no FIV-specific killing in CTL assays with antigen-stimulated PBMC from uninfected cats E266 (Fig. 3A) and AUS3 (Fig. 3B).

Antigen-stimulated effector cells produced an anti-FIV factor(s). As verified in Fig. 1 and 2, the stimulation of PBMC of FIV-infected cats with ConA mitogen induced virus production. However, as expected, the stimulation of PBMC with APC induced major histocompatibility complex-restricted activation of $CD8^+$ T cells, such as the CTL demonstrated in Fig.

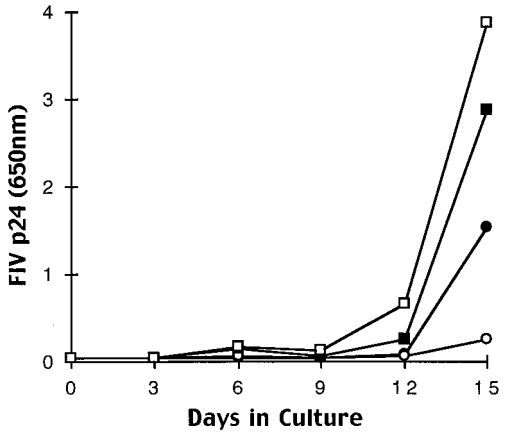

FIG. 2. Kinetics of FIV replication in $CD4^+$ and $CD8^+$ T lymphocytes of infected cats. $CD4^+$ and $CD8^+$ T cells were prepared by the panning method and stimulated with ConA for the first 3 days. Cells were cultured in complete RPMI-1640 supplemented with 100 U of hr IL-2 per ml at a concentration of 5×10^5/ml. FIV replication was measured in the culture supernatants by an ELISA. Data are for FIV-infected cat 306 $CD4^+$ (closed circles) and $CD8^+$ (open circles) T cells and FIV-infected cat 308 $CD4^+$ (closed squares) and $CD8^+$ (open squares) T cells.

3. Virus production in PBMC stimulated with APC was further investigated. PBMC of infected cats were stimulated with autologous, irradiated FIV-infected cells for 3 weeks, and the culture supernatants of stimulated effector cells were collected every 3 or 4 days. In order to study suppressive activity for FIV-PPR replication, the collected supernatants were added to autologous, ConA-stimulated PBMC. ConA-stimulated PBMC readily produced virus after day 12 of culturing, and the amount of virus continued to increase until day 21 of culturing (Fig. 4). After 21 days, virus expression rapidly decreased. In contrast, APC-stimulated PBMC (effector cells) from infected cat 308 produced very little virus (Fig. 4). FIV-PPR replication in APC-stimulated PBMC was inhibited 90% compared with that in ConA-stimulated PBMC following 21 days of culturing without APC. Although the growth rate of APC-stimulated cells for the first 7 days of culturing was slower than that of mitogen-stimulated cells, both types of cells had similar growth rates following the addition of IL-2 to APC-stimulated cells from day 8 to day 25 of culturing (data not shown). The culture supernatants of effector cells also demonstrated inhibition of FIV-PPR replication in autologous, infected ConA-stimulated PBMC (Fig. 4). Virus production from cells cultured with supernatants of effector cells was suppressed 89% compared with that from ConA-stimulated control cells. The growth rate of cells cultured with supernatant was the same as that of control cells cultured without supernatant (data not shown). Although in this experiment the removal of APC from effector cells on day 21 was followed by an increase of viral expression, in additional experiments, the effects of removing APC from effector cells on viral expression were variable (data not shown).

Secretion of a soluble FIV-suppressive factor(s) from antigen-stimulated PBMC of FIV-infected and uninfected cats. In order to confirm that FIV replication could be suppressed by CTL and by the anti-FIV factor(s) in the cell culture supernatants, T-lymphocyte responses were examined with additional uninfected and FIV-infected cats. Cytolytic activity of effector cells from uninfected cats was always less than 1.5%. Although the CTL responses were often low, FIV-specific responses were consistently detected in infected cats (Table 1), suggesting that cytotoxic cellular immune responses are functional in the elimination of virus-infected cells. When FIV-infected cells were cultured with supernatants collected from APC-stimulated effector cells, FIV-PPR replication was inhibited not only by the supernatants of APC-stimulated effector cells from FIV-infected cats but also by the supernatants from uninfected cats (Table 1). In experiment 1, supernatants of stimulated effector cells collected from cats AUO2, AUO3, AZV2, and OLQ5 were shown to significantly suppress viral infection in the PBMC of cat 308. The supernatant of APC-stimulated cells from uninfected cat OLQ4 also demonstrated strong suppressive activity (68% inhibition) for FIV replication. In experiment 2, with three additional FIV-PPR-infected cats, the supernatant from APC-stimulated PBMC of one infected cat, AWF1, demonstrated strong suppression of virus replication. However, the supernatants from APC-stimulated cells of two infected cats, E238 and E284, did not significantly inhibit virus replication. These results confirmed that FIV-PPR replication could be inhibited by a soluble suppressive factor(s) present in the effector cell culture supernatants collected from either FIV-infected or uninfected cats. The soluble anti-FIV factor(s)-mediated inhibitory mechanism was not cytotoxic, be-

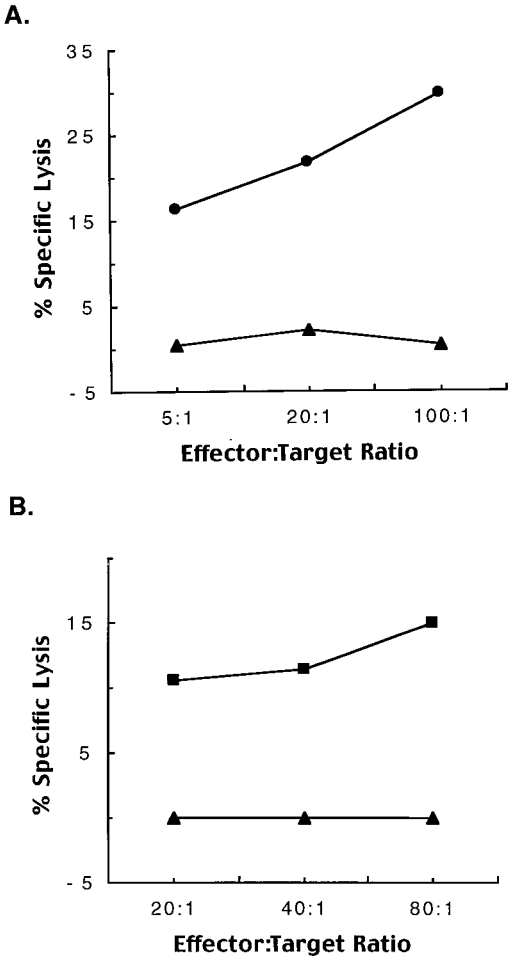

FIG. 3. FIV-specific CTL activities in infected cats. PBMC of FIV-infected and uninfected cats were stimulated in vitro with APC for 2 weeks and used as effector cells. Their cytotoxic activities were measured with ^{111}In-labeled autologous FIV-infected target cells. (A) FIV-infected cat 306 (circles) and uninfected cat E266 (triangles). (B) FIV-infected cat 308 (squares) and uninfected cat AUS3 (triangles).

TABLE 1. CTL and FIV-suppressive responses of uninfected and FIV-infected cats

Expt	FIV status (negative [−] or positive [+])	Cat	CTL response (% lysis)[a]	Virus-suppressive response		
				p24 OD[b]	%	
					Virus expression[c]	Viability[d]
1	−	OLQ4	1.5	0.86 ± 0.10*	32	90.0 ± 3.9
	+	AUO2	8.8	0.43 ± 0.09*	16	91.5 ± 3.4
	+	AUO3	3.0	0.92 ± 0.14*	34	83.2 ± 1.1
	+	AZV2	4.7	0.38 ± 0.06*	14	84.5 ± 10.3
	+	OLQ5	12.5	0.88 ± 0.16*	33	88.9 ± 1.5
	+	308[e]	NA	2.71 ± 0.12	100	90.0 ± 4.6
2	+	E238	50.0	3.77 ± 0.24	117	83.3 ± 3.5
	+	E284	6.8	2.12 ± 0.74	66	83.3 ± 6.0
	+	AWF1	6.0	0.44 ± 0.08*	14	85.0 ± 5.0
	+	284[f]	NA	3.22 ± 0.16	100	86.0 ± 2.0

[a] CTL responses were examined by determining the percent lysis of FIV-infected autologous target cells at E/T ratios of 100:1. Effector cells were stimulated for 14 days by inactivated autologous APC. NA, Not available.
[b] Cell culture supernatants collected from APC-stimulated effector cells for 1 week were added at a medium/supernatant ratio of 1:1 to FIV-PPR-infected cells (cat 308 PBMC) every 3 days. FIV expression was determined after 6 days of culturing by use of an FIV p24 antigen detection ELISA. Data represent the mean ± standard deviation for three experiments. Values marked by an asterisk were statistically different at a P value of <0.05.
[c] Relative to virus expression of untreated control cells.
[d] Viable cells were counted by trypan blue exclusion and reported as the mean ± standard deviation.
[e] These control cells (FIV-PPR-infected cat 308 PBMC) were cultured without effector cell supernatant.
[f] These control cells (FIV-PPR-infected cat 284 PBMC) were cultured without effector cell supernatant.

cause the percent viabilities of the control cells and the cells cultured with supernatants were not statistically different (P, >0.05).

Suppression of FIV replication by the supernatants of antigen-stimulated PBMC but not by the supernatants of ConA-stimulated PBMC. Since FIV-PPR-suppressive activity was demonstrated in the supernatants from APC-stimulated PBMC of uninfected cat OLQ4 (Table 1), additional uninfected cats were used as sources of inducible T lymphocytes. All supernatants collected from APC-stimulated uninfected and FIV-infected cat PBMC strongly inhibited FIV-PPR replication in the infected heterologous cells (P, <0.05) (Table 2).

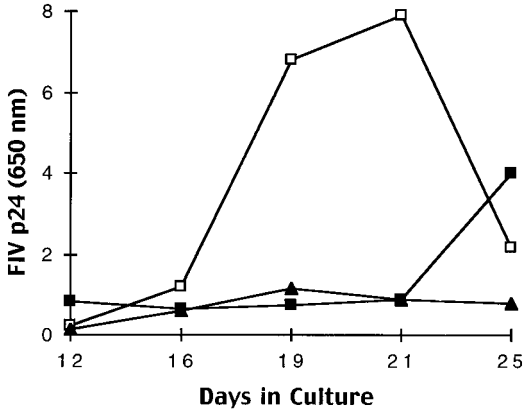

FIG. 4. Suppression of FIV replication in antigen-stimulated effector cells and autologous PBMC cultured with effector cell supernatants. PBMC of FIV-infected cat 308 were divided into three groups. Open squares represent cells stimulated with ConA for the first 3 days and then cultured in normal culture medium. Closed squares represent effector cells stimulated with APC twice a week for 21 days and then cultured for 4 days without APC. The supernatants of effector cells were collected every 3 or 4 days and added to recipient autologous cells. Triangles represent cells stimulated with ConA for the first 3 days and then cultured with the supernatant of stimulated effector cells at a medium/supernatant ratio of 1:1 for 22 days. The supernatants from the three groups of cells were collected every 3 or 4 days, and FIV replication was measured by a capsid antigen ELISA.

However, supernatants obtained from ConA-stimulated PBMC did not demonstrate suppression of FIV-PPR replication (P, >0.05) (Table 2). This result suggested that only antigen-specific stimulation of either FIV-infected or uninfected cat PBMC could induce the secretion of the soluble anti-FIV factor(s) into the supernatants.

TABLE 2. Comparison of FIV-suppressive activities of supernatants collected from antigen- and ConA-stimulated PBMC of FIV-infected and uninfected cats

FIV status	Cat	Results for cell supernatants stimulated by:			
		Antigen[a]		ConA[b]	
		p24 OD[c]	% Virus expression[d]	p24 OD[c]	% Virus expression[d]
Negative	AUS3	1.14 ± 0.08*	31	3.62 ± 0.06	110
	OAE5	1.19 ± 0.25*	32	2.77 ± 0.29	84
	OLM6	2.23 ± 0.20*	61	3.66 ± 0.13	111
	OLQ4	2.36 ± 0.50*	64	3.59 ± 0.23	109
Positive	AUO2	0.60 ± 0.05*	16	3.34 ± 0.28	102
	AUO3	1.40 ± 0.08*	38	3.75 ± 0.05	114
	AZV2	1.82 ± 0.11*	49	3.04 ± 0.30	92
	OLQ5	1.34 ± 0.11*	36	3.50 ± 0.29	106
	308[e]	3.69 ± 0.13	100	3.29 ± 0.22	100

[a] PBMC obtained from uninfected and FIV-infected cats were stimulated with autologous, irradiated APC for 1 week. The collected supernatants from the stimulated cells were added at a medium/supernatant ratio of 1:1 to FIV-PPR-infected cat 308 PBMC.
[b] PBMC obtained from uninfected and FIV-infected cats were stimulated with ConA (5 μg/ml) for 4 days. The collected supernatants from the stimulated cells were added at a medium/supernatant ratio of 1:1 to FIV-PPR-infected cat 308 PBMC.
[c] FIV p24 antigen was measured by use of an ELISA after 6 days of culturing with supernatants. Data represent the mean ± standard deviation for three separate experiments. Values marked by an asterisk were statistically different at a P value of <0.05.
[d] Relative to virus expression of control cells.
[e] These control cells (FIV-PPR-infected cat 308 PBMC) were cultured only with medium.

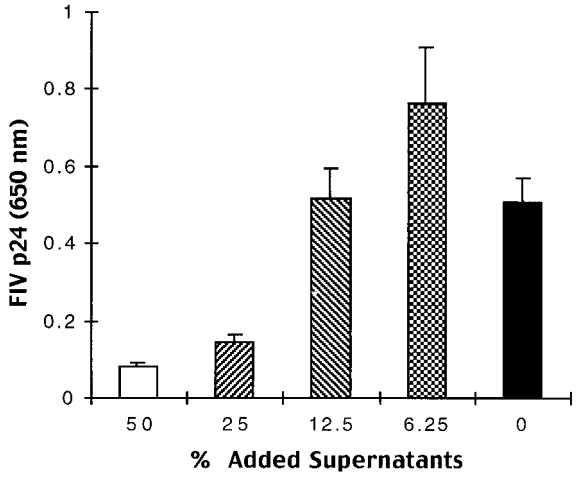

FIG. 5. Dose-dependent activity of the anti-FIV factor(s). Cat 308 PBMC were acutely infected in vitro with FIV and cultured for 4 days with the supernatants of APC-stimulated cat 306 effector cells at concentrations of 50, 25, 12.5, 6.25, and 0%. The supernatants were added to the recipient cells on days 0 and 2 of culturing. FIV replication in the collected supernatants was measured on day 4 by a capsid antigen ELISA. The data are representative of the mean ± standard deviation for three different experiments.

The effect of the anti-FIV factor(s) in the supernatants was dose dependent. The effect of various concentrations of the anti-FIV factor(s) on FIV-PPR replication was examined. The supernatant of cat 306 effector cells was collected after 2 weeks of APC stimulation and added at various concentrations (50, 25, 12.5, 6.25, and 0%) to acutely infected cat 308 PBMC (Fig. 5). The activity of the anti-FIV factor(s) was dose dependent. Cells cultured with 50 and 25% supernatants showed 84 and 71% inhibition of FIV-PPR replication, respectively. However, cells cultured with 12.5% supernatant did not suppress viral expression at all. Interestingly, viral replication in cells cultured with 6.25% supernatant was increased.

Cross-reactivity and cell specificity of the anti-FIV factor(s). The cross-reactive suppression of the anti-FIV factor(s) was examined with FIV-PPR and FIV-Pet. The supernatant collected from FIV-PPR-infected cat PBMC which had been stimulated with irradiated FIV-PPR-infected APC suppressed viral replication in FIV-Pet-infected PBMC (Fig. 6A). There was 96.3% inhibition of viral replication in supernatant-treated cells compared to nontreated control cells. This result demonstrated that the suppressive activity of the anti-FIV factor(s) was not strain specific. The same supernatant containing the anti-FIV factor(s) used in the experiment shown in Fig. 6A was examined for the suppression of FIV-Pet infection in CrFK cells (Fig. 6B). However, the anti-FIV factor(s) did not suppress FIV-Pet expression in infected CrFK cells. Therefore, the anti-FIV factor(s) produced from APC-activated PBMC suppressed the replication of FIV-PPR and FIV-Pet in PBMC but not the replication of FIV-Pet in CrFK cells.

The anti-FIV factor(s) originated from $CD8^+$ T lymphocytes. In order to determine the phenotype of the T cells producing the anti-FIV factor(s), $CD4^+$ or $CD8^+$ T cells were depleted from PBMC stimulated for 7 days with APC. The enriched $CD8^+$ or $CD4^+$ T cells were cultured for an additional 7 days with APC, and the culture supernatants were collected. The supernatants were added to FIV-PPR-infected cat 308 PBMC, and their suppressive activities were examined after 12 days of culturing (Fig. 7). The infected cells cultured with the supernatant of cat 306 $CD8^+$ T cells demonstrated

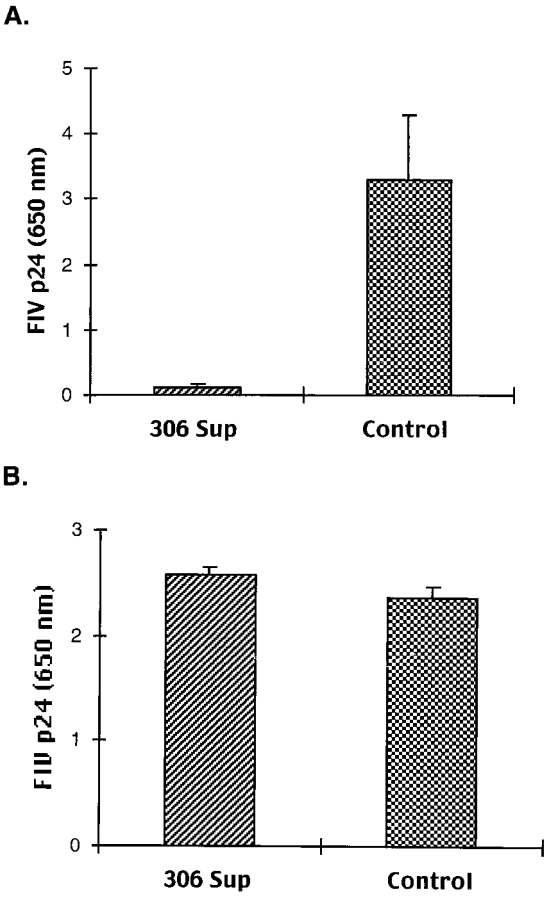

FIG. 6. (A) Cross-reactivity of the anti-FIV factor(s). PBMC infected in vitro with FIV-Pet for 10 days were cultured for 11 days with supernatant collected from FIV-PPR-infected cat 306 PBMC (306 Sup) which had been stimulated with autologous APC for 2 weeks. Control cells were cultured with complete RPMI 1640. Supernatant and medium were added to the infected cells every 3 days. FIV replication determined by an FIV p24 ELISA for cells cultured with supernatant was compared with that for control cells cultured without supernatant. The data are representative of three different experiments. (B) Cell specificity of the anti-FIV factor(s). Chronically FIV-Pet-infected CrFK cells (10^5/ml) were cultured for 4 days with supernatant collected from FIV-PPR-infected cat 306 PBMC (306 Sup) which had been stimulated with autologous APC for 2 weeks. Control cells were cultured with complete DMEM. Supernatant and medium were added to cells every 2 days. FIV replication determined by an FIV capsid antigen ELISA for cells cultured with supernatant was compared with that for control cells cultured without supernatant. The data are representative of three separate experiments. Error bars in both panels indicate standard deviations.

82.8% inhibition of viral replication, compared with the infected control cells. The supernatant of cat 308 $CD8^+$ T cells demonstrated 46.5% suppression of viral replication in infected cells. However, no inhibition of FIV replication was observed in cells cultured with the supernatant of 308 $CD4^+$ T cells.

The soluble anti-FIV factor(s) shares suppressive activity with human chemokine MIP-1α. It is known that several human chemokines have HIV-suppressive activity (6, 13). The FIV-suppressive activity of the supernatant of stimulated cat 306 PBMC was compared with those of human recombinant β-chemokines, MIP-1α, MIP-1β, and RANTES, and an α-chemokine, SDF-1α (Table 3). Human MIP-1α, as well as the cat 306 supernatant, suppressed FIV replication (P, <0.05) in FIV-PPR-infected cells. SDF-1α, even at a concentration of 2,000 ng/ml, did not significantly (P, >0.05) inhibit FIV-PPR

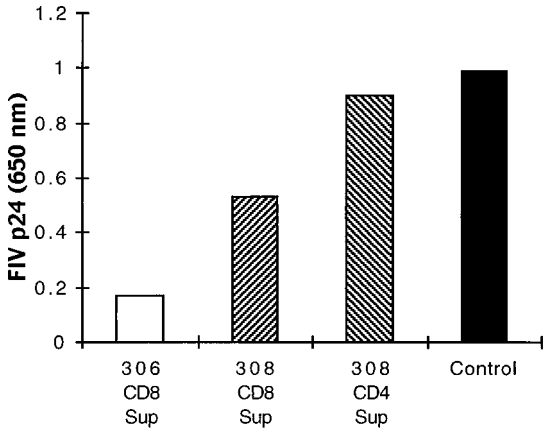

FIG. 7. Production of the anti-FIV factor(s) from $CD8^+$ T cells. After 7 days of stimulation of PBMC of FIV-infected cats 306 and 308 with APC, $CD4^+$ or $CD8^+$ T cells were depleted by the panning method. Unbound $CD8^+$ or $CD4^+$ T cells were collected. The separated $CD8^+$ and $CD4^+$ T cells were stimulated again with APC for 7 days, and the supernatants (Sup) were collected. FIV-infected cat 308 PBMC were cultured with the supernatants from 306 $CD8^+$, 308 $CD8^+$, and 308 $CD4^+$ T cells for 12 days. The supernatants were added to the recipient cells on days 0, 3, 6, and 9 of culturing. FIV replication was measured on day 12 by an ELISA. The data are representative of two separate experiments.

replication. In a separate experiment, 500 ng of SDF-1α actually resulted in expression of FIV that was 130% that in untreated control cells (data not shown). The percent viabilities of FIV-infected cells cultured with MIP-1α and cat 306 supernatant, which were 91.2 and 92.0%, respectively, did not differ from that of the control cells (P, >0.05). Therefore, it was demonstrated that the FIV inhibition mediated by the anti-FIV factor(s) produced from APC-stimulated feline PBMC and human MIP-1α was noncytolytic.

DISCUSSION

Although, unlike HIV, FIV readily infects both $CD4^+$ and $CD8^+$ T lymphocytes (9), $CD4^+$ cells have been reported to have the greatest proviral burden in vivo in acutely FIV-infected cats (14, 15). In contrast, reverse transcriptase activities in the supernatants of cloned $CD4^+$ T cells were not necessarily higher than those in $CD8^+$ T cells after in vitro infection (9). In this study, it was found that $CD4^+$ T cells of one FIV-infected cat produced more virus than autologous $CD8^+$ T cells. However, $CD8^+$ T cells of a second FIV-infected cat produced more virus than $CD4^+$ T cells. Therefore, both feline $CD4^+$ and $CD8^+$ T cells can be major reservoirs for FIV-PPR in chronically infected cats.

Protective in vivo immune responses have been associated with the presence of FIV-specific CTL (18, 45). FIV-specific CTL could be induced from PBMC of infected cats by in vitro stimulation with autologous APC, whereas no FIV-specific CTL activities were observed in PBMC of uninfected cats under the same stimulation conditions (3, 40). FIV-specific CTL could contribute to the control of virus replication in vivo, thus maintaining the clinically healthy state of FIV-infected cats. In this study, APC-stimulated effector cells of FIV-infected cats consistently demonstrated CTL activity. Although the activity was often below 10%, the CTL response of infected cats was consistently greater than the cytolytic response of effector cells from uninfected cats.

The amount of viral replication in PBMC from infected cats cocultured with APC was found to be significantly lower than the amount of virus detected in mitogen-stimulated PBMC from the same cats. These studies focused on the nature of this apparent suppression of FIV infection. FIV replication in PBMC cultured without APC stimulation was 10-fold higher than that in antigen-stimulated effector cells. Therefore, a population of PBMC, some of which are $CD8^+$ T lymphocytes with integrated FIV proviral DNA, produces viral particles in the presence of mitogenic activation. However, in the presence of virus-specific antigen stimulation, $CD8^+$ T lymphocytes may develop unidentified antiviral activities that in themselves would inhibit endogenous viral replication. In addition, it has been shown that FIV-specific APC stimulation increases the relative number of $CD8^+$ T cells (28). Likewise, we observed that APC stimulation induced an increase in the numbers of $CD8^+$ cells and a decrease in the numbers of $CD4^+$ cells (data not shown).

$CD8^+$ T cells of HIV-infected patients suppressed HIV replication when they were added to $CD8^+$ T-cell-depleted PBMC. It was suggested that this antiviral activity was noncytolytic and mediated by a soluble factor (8, 47, 48). The $CD8^+$ T-cell-mediated noncytotoxic antiviral activity was also suggested to correlate with the clinically healthy or uninfected state of HIV-infected or HIV-exposed individuals (4, 27, 43). $CD8^+$ T-cell-mediated suppression of HIV replication by $CD8^+$ T cells obtained from HIV-uninfected human beings and chimpanzees was also demonstrated (12, 25). Similarly, the FIV-specific $CD8^+$ T-lymphocyte antiviral activity in infected cats was also demonstrated by coculturing $CD8^+$ with FIV-infected $CD4^+$ T or MYA-1 cells, reducing viral replication in the infected cells (10, 19, 23). In the present study, the anti-FIV factor(s) could be produced from both FIV-infected and uninfected cat PBMC. The $CD8^+$ T lymphocytes did not require prior exposure to a specific antigen for the production of an APC-inducible anti-FIV factor(s). These distinct functions may represent distinct subsets of $CD8^+$ T lymphocytes. The CTL responses of infected cats did not correlate with the level of suppression mediated by the anti-FIV factor(s) observed for each animal. In fact, whereas the APC-exposed PBMC of cats E238 and E284 did not produce suppressive activity for FIV-infected lymphocytes, E238 exhibited the most impressive CTL

TABLE 3. Comparison of FIV-suppressive activities of anti-FIV factor(s)-containing supernatants and human chemokines

Treatment	p24 OD[a]	%	
		Virus expression[b]	Viability[c]
MIP-1α	0.13 ± 0.03*	48	91.2 ± 4.6
MIP-1β	0.27 ± 0.12	102	88.8 ± 5.3
RANTES	0.24 ± 0.07	89	93.5 ± 5.1
SDF-1α	0.17 ± 0.07	66	93.3 ± 2.0
Cat 306 supernatant	0.07 ± 0.01*	28	92.0 ± 4.1
Control cells[d]	0.26 ± 0.05	100	87.5 ± 1.6

[a] Determined by use of an FIV p24 antigen detection ELISA for cell culture supernatants after FIV-PPR-infected cat 306 PBMC were cultured for 6 days with human β-chemokines (MIP-1α, MIP-1β, and RANTES), a human α-chemokine (SDF-1α), and APC-stimulated effector cell supernatant (cat 306 supernatant). The concentration of human MIP-1α, MIP-1β, and RANTES was 500 ng/ml, and that of human SDF-1α was 2,000 ng/ml. Cat 306 supernatant was added at a medium/supernatant ratio of 1:1. Data represent the mean ± standard deviation for three separate experiments. Values marked by an asterisk were statistically different at a P value of <0.05.

[b] Relative to virus expression of control cells.

[c] Viable cells were counted by trypan blue exclusion and reported as the mean ± standard deviation.

[d] Control cells (FIV-PPR-infected cat 306 PBMC) were cultured only with medium.

response of any cat infected with FIV-PPR that we have examined.

Only cats E238 and E284 failed to produce inducible suppressive activity. Whereas all other cats in this study had initially been infected with either 50 or 250 50% tissue culture infective doses of FIV-PPR, these cats received 1,250 50% tissue culture infective doses. The pathogenesis may have been more severe, because the transient central nervous system illness was more pronounced in these two cats (unpublished data). It will be of interest to further evaluate differences between these animals and those that readily produced the suppressive activity.

Induction of the suppressive activity by T lymphocytes may be the result of a direct interaction with APC or, alternatively, the result of an indirect mechanism, such as the release of cytokines by APC. The major difference between the suppressive responses described in this study and most studies examining $CD8^+$ T-cell-mediated antiviral activity is that in this study, supernatants containing the anti-FIV factor(s) were obtained from APC-stimulated effector cells rather than from nonspecific mitogen-stimulated cells. However, the soluble anti-FIV dose-responsive factor(s) demonstrated in this study is similar to the previously described $CD8^+$ T-cell-mediated anti-HIV factor (8, 30). The anti-FIV factor(s) suppressed viral replication without eliminating infected cells.

To date, several factors have been shown to inhibit lentivirus replication. The chemokines RANTES, MIP-1α, MIP-1β, MDC, and SDF-1α have been shown to inhibit HIV replication (6, 13, 33), and IL-16 has been shown to suppress the replication of HIV and SIV (2). CAF, a $CD8^+$ T-lymphocyte product that differs from IL-16 and the described chemokines, may be closely related to the feline $CD8^+$ T-lymphocyte factor (24, 29, 30, 34). It was suggested that several antiviral factors, each with distinct activity, could be involved in the suppression of virus replication (38, 39).

The human β-chemokines RANTES, MIP-1α, MIP-1β, and MCP-1 could not prevent FIV-Pet infection in CrFK cells or in T lymphocytes (22). In the present study, we demonstrated the suppression of FIV-PPR replication in feline PBMC by human MIP-1α at a concentration of 500 ng/ml, but equivalent concentrations of the other β-chemokines tested did not suppress. Although the α-chemokine SDF-1α seemed to suppress FIV replication at a concentration of 2,000 ng/ml, the suppression was not statistically significant ($0.1 > P > 0.05$). When 500 ng of SDF-1α per ml was used with infected PBMC, FIV expression was actually increased rather than suppressed (data not shown). Human SDF-1α has been shown to inhibit FIV-Pet infection in CrFK cells but not in T-cell lines, depending on the incubation conditions (22). Two FIV strains, FIV-PPR and FIV-Pet, have 91% homology in their amino acid sequences; however, they have distinct cell tropisms and replication kinetics in feline PBMC (36). The anti-FIV factor(s) demonstrated in this study could inhibit both FIV-PPR and FIV-Pet replication in PBMC. However, the FIV-suppressive factor(s) could not suppress FIV-Pet replication in CrFK cells, suggesting cell specificity. Therefore, the anti-FIV factor(s) seems to differ fundamentally from SDF-1α.

The exact nature of the anti-FIV factor(s) produced by stimulated feline PBMC and the factor-secreting mechanism remain to be further defined. One factor or a combination of factors, either known or at present unidentified, could be responsible for inhibiting FIV replication in lymphocytes. Further examination of the mechanism responsible for the induction of suppressive activity and the mechanism of viral suppression could lead to a better understanding of the role of $CD8^+$ T cells in viral pathogenesis.

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